APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1995, p. 3069–3075 Vol. 61, No. 8
0099-2240/95/$04.00+0
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Quantitative Fluorescence In Situ Hybridization of *Bifidobacterium* spp. with Genus-Specific 16S rRNA-Targeted Probes and Its Application in Fecal Samples

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Received 21 February 1995/Accepted 26 May 1995

Three 16S rRNA hybridization probes were developed and tested for genus-specific detection of *Bifidobacterium* species in the human fecal flora. Variable regions V2, V4, and V8 of the 16S rRNA contained sequences unique to this genus and proved applicable as target sites for oligodeoxynucleotide probes. Determination of the genus specificity of the oligonucleotides was performed by whole-cell hybridization with fluorescein isothiocyanate-labelled probes. To this end, cells were fixed on glass slides, hybridized with the probes, and monitored by videomicroscopy. In combination with image analysis, this allowed quantification of the fluorescence per cell and objective evaluation of hybridization experiments. One of the probes developed was used to determine the population of *Bifidobacterium* spp. in human fecal samples. A comparison was made with results obtained by cultural methods for enumeration. Since both methods gave similar population estimates, it was concluded that all bifidobacteria in feces were culturable. However, since the total culturable counts were only a fraction of the total microscopic counts, the contribution of bifidobacteria to the total intestinal microflora was overestimated by almost 10-fold when cultural methods were used as the sole method for enumeration.

The human intestinal tract harbors an active and complex bacterial ecosystem. The composition and activity of this indigenous gut flora are of paramount importance to human immunology, nutrition, and pathogenesis and hence to the health of the individual (29). Colonization resistance, or bacterial antagonism of the indigenous intestinal microflora, for example, represents a first line of defense against the establishment of pathogenic microorganisms in the intestinal tract (28, 29). The population structure of the intestinal flora influences the effectiveness of colonization resistance greatly (28). Traditional methods to determine the composition of the microflora require cultivation on selective media, which is laborious, time-consuming, and prone to statistical and methodological error. For this reason, the dynamics of the population composition are difficult to monitor, which hampers objective evaluation of gut flora modulation studies.

In recent years, 16S rRNA probe hybridization has become widely adopted for detection of specific bacterial groups in mixed populations (2, 3, 24, 25, 30, 31). This method is based on the hybridization of synthetic oligodeoxynucleotide probes to specific regions within the bacterial ribosome and does not require cultivation. The specificity of the probe can be adjusted to fit any taxonomic ranking, from kingdom to subspecies (1, 9, 20). DNA hybridization probes have been applied successfully to the intestinal ecosystem. In human feces, *Bacteroides vulgatus* can be detected by hybridization with a chromosome-targeted probe (12), and in the feces of mice, 16S rRNA-targeted probes have been shown to be applicable for the detection of *Fibrobacter intestinalis* (1). Species-specific 16S rRNA probes have been developed for five *Bifidobacterium* spp. of the human intestinal microflora (35), but the in situ application of these probes has not yet been reported.

In the human intestinal microflora, *Bifidobacterium* is the third most common genus after the genera *Bacteroides* and *Eubacterium* (7). The potential use of bifidobacteria in the treatment and prevention of gastrointestinal disorders (18) has raised the demand for an accurate and easy method for their detection and enumeration. In contrast to the genera *Bacteroides* and *Eubacterium*, the bifidobacteria form a monophyletic cluster on the basis of 16S rRNA sequences (13). This facilitates the development of genus-specific 16S rRNA probes. In studies of the compositional dynamics and metabolic activity of the complete gut flora, genus-specific probes for the identification of distinct physiological groups would combine rapid monitoring with useful taxonomic resolution; i.e., when applied in concert, a limited number of genus-specific probes could be used to describe the overall composition and metabolic potential of the total population. In this communication, we report the development of genus-specific 16S rRNA hybridization probes for the detection of bifidobacteria in human fecal samples.

For the detection of fluorescent oligonucleotide probes hybridized to bacteria on microscopic slides, photography can be applied. However, quantification by this method is hampered by the absence of an objective threshold criterion for discrimination between hybridized and nonhybridized cells. Therefore, for objective evaluation of probe specificity, we have employed an image analysis system which allows fluorometry of individual cells (32–34). In the present study, we have compared 16S rRNA hybridization with classical cultivation techniques for the quantification of bifidobacteria in human fecal samples. The results indicate that virtually all bifidobacteria

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TABLE 1. Probes used in this study

Probe	Target[a]	Sequence[b] (5′→3′)	T_d[c] (°C)
Uni519	519–536	GTATTACCGCGGCTGCTG	60.2
Bif164	164–181	CATCCGGCATTACCACCC	60.2
Bif662	662–679	CCACCGTTACACCGGGAA	60.2
Bif1278	1278–1294	CCGGTTTTCAGGGATCC	56.6

[a] Target region for hybridization in the 16S rRNA molecule, numbered according to the homologous *E. coli* sequence (6).
[b] Sequence from 5′ to 3′ end. The 5′ end was FITC labelled.
[c] T_d, theoretical dissociation temperature, based on the formula $T_d = 81.5 + 16.6 \log[Na^+] + 0.41(\%G+C) - 820/(\text{probe length})$, according to reference 13.

present in feces can form colonies on the *Bifidobacterium*-selective agar medium employed.

MATERIALS AND METHODS

Probes for in situ hybridization. The 16S rRNA sequences of 18 *Bifidobacterium* spp. were retrieved from the EMBL and GenBank data libraries (4, 21). Among these were nine *Bifidobacterium* species which had been isolated from human fecal samples (22). After comparison of unique bifidobacterial sequences with a large number of homologous reference sequences (13), two potential target regions for hybridization probes were selected. The sequences and target sites of the probes are presented in Table 1. Probes Bif164 and Bif662 are complementary to sites in variable regions V2 and V4, respectively (17). A third probe, Bif1278, directed against a site in region V8, was suggested by Frothingham et al. (8). The universal-kingdom probe Uni519 (9) served as a positive control. The oligonucleotide probes were commercially synthesized and were 5′ end labelled with fluorescein isothiocyanate (FITC; Pharmacia Biotech).

Maintenance of cultures. Bacterial cultures were maintained in anoxic chopped meat carbohydrate (CMC) medium (10) at room temperature, with bimonthly transfers, and in anoxic skimmed milk at −20°C. Prior to hybridization, fresh cultures were grown in anoxic peptone-yeast extract-glucose (PYG) medium (10) at 37°C.

Testing of probe specificity. The bacterial species used for testing the specificity of the probes were derived from human and animal fecal and clinical samples. The probe specificities were examined with 16 species of the genus *Bifidobacterium* (Table 2) and 20 reference species that are common inhabitants of the human intestinal tract (Table 3). Exponentially growing cells were fixed with 3% (vol/vol) formaldehyde. After 10 min of storage at 4°C, 10 μl of the fixed-cell suspension was applied to a glass slide that had been treated with Vectabond (Vector Laboratories, Burlingame, Calif.) to ensure proper adhesion of the cells. After drying for 20 min at 45°C, the dried cell smears were dehydrated in a graded ethanol series (50, 75, and 98% ethanol [vol/vol], 2 min). Twelve microliters of 0.2-μm-pore-size-filtered hybridization buffer was added to the cell smears. The hybridization buffer consisted of 0.9 M NaCl, 20 mM Tris-HCl (pH 7.5), and 0.1% (wt/vol) sodium dodecyl sulfate. After addition of 8 ng of FITC-labelled probe per μl, the smears were covered with a coverslip. The slides were incubated in a buffer-saturated hybridization chamber at 45°C for 15 to 20 h. After hybridization, the slides were washed for 15 min in 50 ml of hybridization buffer at 45°C and air dried, and the cell smears were embedded in mounting fluid, consisting of a 1:1 mixture of glycerol and phosphate-buffered saline (PBS; 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 per liter) supplemented with 2.5% (wt/vol) NaI.

TABLE 2. *Bifidobacterium* species used in this study

Bifidobacterium species	Source[a]	Present in human feces[b]
B. adolescentis	NIZO B659	+
B. angulatum	NIZO B664	+
B. animalis	LMG 3303	−
B. asteroides	NIZO B657	−
B. bifidum	ATCC 29521	+
B. boum	NIZO B665	−
B. breve	LMG 3035	+
B. cornutum	LMG 3268	+
B. dentium	ATCC 27678	+
B. globosum	ATCC 25865	−
B. infantis	ATCC 15697	+
B. longum	LMG 3277	+
B. magnum	NIZO B668	−
B. pseudolongum	NIZO B669	−
B. subtile	NIZO B672	−
B. suis	NIZO B673	−

[a] NIZO, Netherlands Institute for Dairy Research, Ede, The Netherlands; LMG, Department of Medical Microbiology, University of Groningen, Groningen, The Netherlands; ATCC, American Type Culture Collection, Rockville, Md.
[b] According to reference 22.

TABLE 3. Reference strains of intestinal bacteria used in this study

Species	Source[a]
Bacteroides distasonis	LMG 3335
Bacteroides fragilis	LMG 3407
Bacteroides thetaiotaomicron	RPHL 2
Bacteroides uniformis	LMG 3435
Bacteroides vulgatus	RPHL 1
Clostridium butyricum	LMG 3316
Clostridium indolis	LMG 3323
Clostridium innocuum	LMG 3285
Clostridium nexile	LMG 3333
Clostridium perfringens	LMG HB100
Escherichia coli	LMG EPI1
Eubacterium cylindroides	LMG 3291
Eubacterium ruminantium	LMG 3367
Eubacterium sp.	RPHL 4
Fusobacterium mortiferum	LMG HB120
Fusobacterium vanium	LMG HB110
Lactobacillus acidophilus	DSM 20077
Lactobacillus sp.	RPHL 5
Peptostreptococcus anaerobius	LMG HB700
Peptostreptococcus asaccharolyticus	LMG HB800

[a] LMG, Department of Medical Microbiology, University of Groningen, Groningen, The Netherlands; RPHL, Regional Public Health Laboratory, Leeuwarden, The Netherlands; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

The slides were evaluated with an Orthoplan epifluorescence microscope (Leitz) equipped with a mercury arc lamp (HBO100W/2; Osram, Alphen aan den Rijn, The Netherlands), a 50× PL Fluotar objective (Leitz), an I2/3 (blue excitation) filter block, and a Peltier cooled charge-coupled device (CCD) video camera (Loral Fairchild CCD 5000/1; Loral Fairchild, Sunnyvale, Calif.). An exposure extension system for this camera was described previously (33). The image analysis software used was the Groningen Reduction of Image Data (GRID) system (14, 34). The fluorescence measurements were performed with the immunofluorescence package described previously (11). Surface fluorescence signals were calibrated by using a nonfading uranyl glass reference (32). Phase-contrast and fluorescence images of each field of view were obtained. The shape of each bacterium was determined from the phase-contrast image automatically. The fluorescence of each object was determined from the corresponding area in the fluorescence image. This procedure avoids exclusion of nonfluorescent objects. The fluorescent images were recorded with a camera exposure time of 12 s. Per microscopic slide, 250 to 500 objects were measured. For each slide, the negative control (autofluorescence) was determined, and the 95th percentile of the fluorescence distribution served as a threshold. The percentage of positively hybridized objects, with fluorescence above the threshold, per total number of objects detected under phase-contrast illumination was dubbed the hybridization percentage. This percentage was determined to evaluate the performance of the probes and was done on pure cultures. For graphic representation, surface fluorescence intensity was plotted versus adaptive kernel estimates of the probability density distribution (5) of individual objects, and the maxima of probability density were scaled to 1.00 for presentation purposes.

Enumeration of bifidobacteria and total anaerobes in feces. For quantification of bifidobacteria and total anaerobes in feces, stool specimens from 10 healthy human volunteers were collected and processed as described previously (15). The cultural counts of bifidobacteria were determined on prereduced agar-solidified *Bifidobacterium* medium (BIF) (27). Since this medium is not highly selective, only those colony types that hybridized positively with probe Bif164 in a separate colony hybridization test were counted. Per colony type, these tests were performed in triplicate with cell smears on glass slides as described above. To improve stringency, the hybridization and washing temperature was increased to 50°C. The total number of culturable anaerobes was assessed on prereduced (nonselective) brucella blood agar (BBA) prepared from brucella agar base (Oxoid, Basingstoke, England) supplemented with 5% (vol/vol) sheep blood, 1 μg of vitamin K_1 per ml, and 5 μg of hemin per ml as described before (26). Cultural counts (CFU) of bifidobacteria and total anaerobes were determined in triplicate.

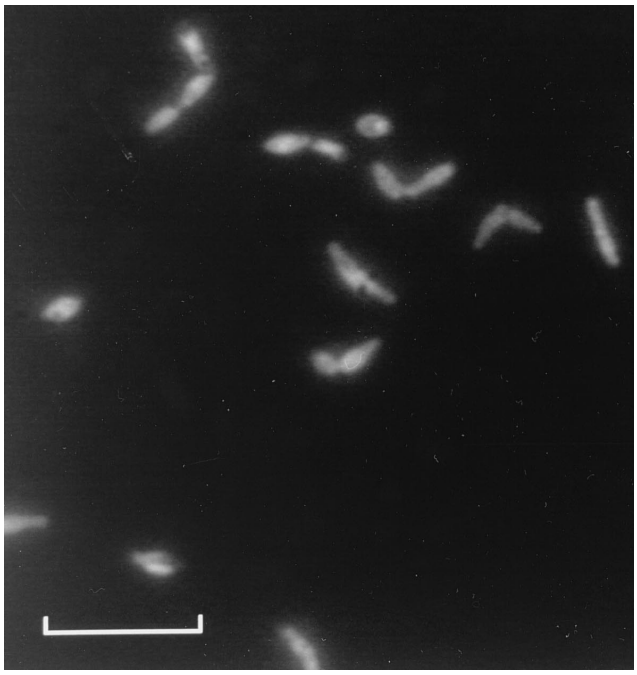

FIG. 1. *B. longum* hybridized with probe Bif164 at 45°C on a glass slide, recorded by the GRID image processing system. Image file printed directly on Kodak T-MAX 100 B/W film, with a Lasergraphics LFR Mark II laserfilm recorder. The fluorescing cells are well separated from the background. Bar, 10 μm.

With the same fecal samples, bifidobacteria were quantified by fluorescence in situ hybridization with the genus-specific probe Bif164 at 50°C. One gram of homogenized feces was suspended in 9 ml of 0.2-μm-pore-size-filtered PBS. This suspension was diluted 10 times in filtered PBS and thoroughly mixed. After removal of debris (35 × *g*, 15 min), the supernatant was collected and fixed overnight at 4°C with 4% (wt/vol) fresh paraformaldehyde solution. Cells from 1 ml of fixed-cell suspension were washed twice (8,000 × *g*, 5 min) in 1 ml of filtered PBS and resuspended in 1 ml of a mixture of PBS and ethanol (1:1). After 1 h of storage at −20°C, 5 μl of cell suspension was added to 50 μl of warmed (50°C) hybridization buffer, and 5 μl of FITC-labelled probe was added. Cells were hybridized for 40 h at 50°C. After resuspension in 1 ml of hybridization buffer, cells were filtered on a 0.2-μm-pore-size Isopore polycarbonate membrane filter (Millipore Corporation) and washed twice with 10 ml of warm (50°C) hybridization buffer. Filters were mounted on microscope slides with Vectashield, and hybridized cells were counted visually with an Olympus BH2 microscope with a DPlanApo100UVPL objective (100×; numerical aperture, 1.30), an HBO100W/2 mercury lamp, and an IB blue light excitation block with an extra EY455 excitation filter. The total number of bacteria present in feces was determined by the method of Porter and Feig (19), with 0.5 μg (wt/vol) of 4′,6-diamidino-2-phenylindole (DAPI) as a DNA stain under illumination with a UV excitation filter block and by the direct microscopic clump count (DMCC) method (10). During Gram stain preparation for DMCC counts, very mild washings were applied. All microscopic counts were determined in duplicate, with a minimum of 300 cells counted per assay. Statistical analysis included the Student *t* test and *F* test.

RESULTS

Selection of target regions. The proposed probe Bif1278 contained one mismatch for *B. adolescentis*, *B. coryneforme*, and *B. cunniculi*. All non-*Bifidobacterium* species in the RDP database (13) had at least three mismatches. Probe Bif662 was complementary to the 16S rRNA of all *Bifidobacterium* species. Three *Chlorobium* species and *Gardnerella vaginalis* contained one mismatch, and several bacterial species had two mismatches for this probe. None of these bacteria represented known inhabitants of the human intestinal tract. All other sequences in the RDP database contained three or more mismatches for probe Bif662. Probe Bif164 matched with 16 *Bifidobacterium* spp. in the database. It had one mismatch for

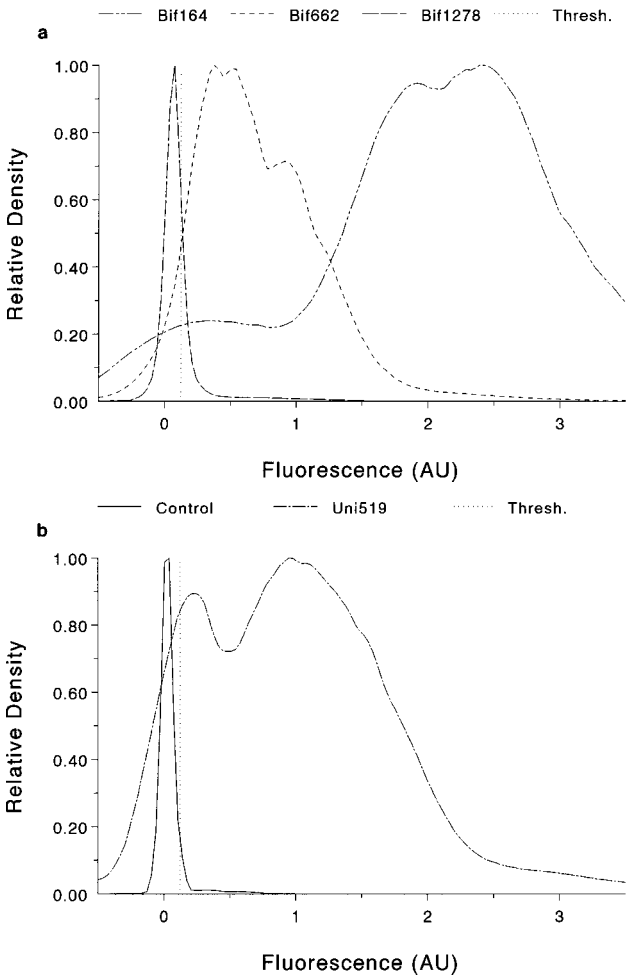

FIG. 2. Fluorescence in situ hybridization with *B. adolescentis*. (a) Distribution of fluorescence intensity of individual cells as obtained with the three *Bifidobacterium*-specific probes. (b) Distribution of fluorescence intensity of negative control and positive control. Threshold levels are indicated by vertical dotted lines. AU, arbitrary units.

B. coryneforme, *B. cunniculi*, *Pseudomonas diminuta*, and *G. vaginalis*, but none of these species are normally encountered in human intestinal flora (7, 22). Furthermore, this probe had two mismatches for *Bacillus cycloheptanicus* and three mismatches for a number of bacteria, none of which were known representatives of the human intestinal flora. All other organisms in the RDP database had more than three mismatches with probe Bif164.

Probe specificity. After whole-cell hybridization and recording by the CCD camera, fluorescence images, as shown in Fig. 1, were obtained. The fluorescence within the cells was clearly distinct from the dark background and was readily quantifiable by the GRID system. With these quantitative data, a probability density distribution as a function of the fluorescence intensity of individual cells was estimated. Figure 2 is an example of such a plot. In this case, *B. adolescentis* was hybridized with all three *Bifidobacterium*-specific probes (Fig. 2a). The fluorescence distribution obtained with probes Bif164 and Bif662 could readily be distinguished from that of the negative control (Fig. 2b). The distribution obtained with probe Bif1278, which contains one mismatch for *B. adolescentis*, could not be distinguished from that of the negative control. The mean

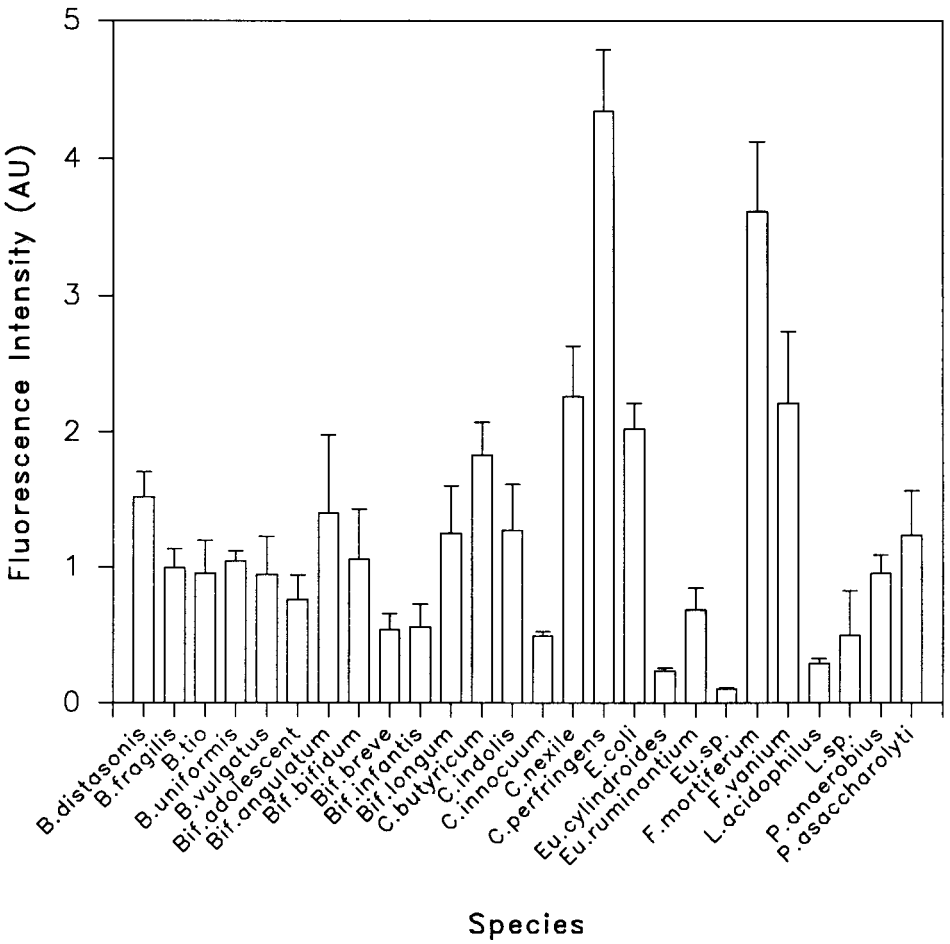

FIG. 3. Comparison of the median values of the fluorescence distribution of several species of intestinal bacteria after in situ hybridization with probe Uni519. B., *Bacteroides*; Bif., *Bifidobacterium*; C., *Clostridium*; E., *Escherichia*; Eu., *Eubacterium*; F., *Fusobacterium*; L., *Lactobacillus*; P., *Peptostreptococcus*. The species tested are listed in Tables 2 and 3.

fluorescence signals (± standard error of the mean [SEM]) for all *Bifidobacterium* spp. hybridized at 45°C with probes Bif164, Bif662, Bif1278, Uni519, and the negative control were 1.381 ± 0.182, 0.866 ± 0.199, 0.215 ± 0.029, 0.919 ± 0.098, and 0.075 ± 0.011, respectively.

The hybridization percentages offer a more objective measure for analysis of probe specificities. Pure cultures of the *Bifidobacterium* spp. that are important members of the human fecal flora, i.e., *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. infantis*, and *B. longum*, exhibited hybridization percentages with Bif164 at 45°C that exceeded 81%. These values were similar to those obtained with Uni519. With probe Bif662, *B. infantis* and *B. breve* exhibited hybridization percentages of 34 and 59%, respectively. The other bifidobacteria from human feces yielded hybridization percentages of greater than 68% with this probe at this stringency. Probe Bif1278 yielded low fluorescence levels, resulting in low hybridization percentages. For bifidobacteria, hybridization percentages with probe Bif164 were significantly higher than with probes Bif662 and Bif1278. For all of the 16 *Bifidobacterium* spp. tested at 45°C, the mean hybridization percentages (± SEM) with probes Bif164, Bif662, Bif1278, and Uni519 were 83% ± 2%, 73% ± 4%, 34% ± 4%, and 83% ± 2%, respectively. Exceptions were *B. cornutum* and *B. globosum*, which showed extremely low fluorescence after hybridization with all probes, including the universal probe. Another species, *B. magnum*, showed an exceptionally low hybridization percentage only with probe Bif164 (37%). Hybridization of the *Bifidobacterium* probes with non-*Bifidobacterium* spp. at 45°C resulted in levels of fluorescence comparable to the negative control values.

The level of fluorescence obtained with Uni519 varied considerably between species (Fig. 3). Although the universal probe resulted in low levels of fluorescence relative to values obtained with *Bifidobacterium*-specific probes, the hybridization percentage of most species tested was more than 80% (Fig. 4). The mean hybridization percentage of nonbifidobacteria with probes Bif164, Bif662, and Bif1278 at 45°C was less than 10%, on average. A typical result, such as that for *Bacteroides* spp., yielded hybridization percentages of 8, 15, and 15% with Bif164, Bif662, and Bif1278, respectively (Fig. 4). The universal probe Uni519 resulted in a value of 91% in this case.

Quantification of bifidobacteria in feces. Colony hybridization tests with probe Bif164 showed that BIF agar was indeed not fully selective for bifidobacteria when culturing fecal samples. The number of non-*Bifidobacterium* colonies that developed on BIF agar varied between the samples and was generally between 0 and 10% of the total number of CFU. The mean number (± SE) of culturable bifidobacteria from all samples was 2.45 (± 1.40) × 10^9 per g of wet feces (Table 4). The mean number of total culturable anaerobes on BBA from all sam-

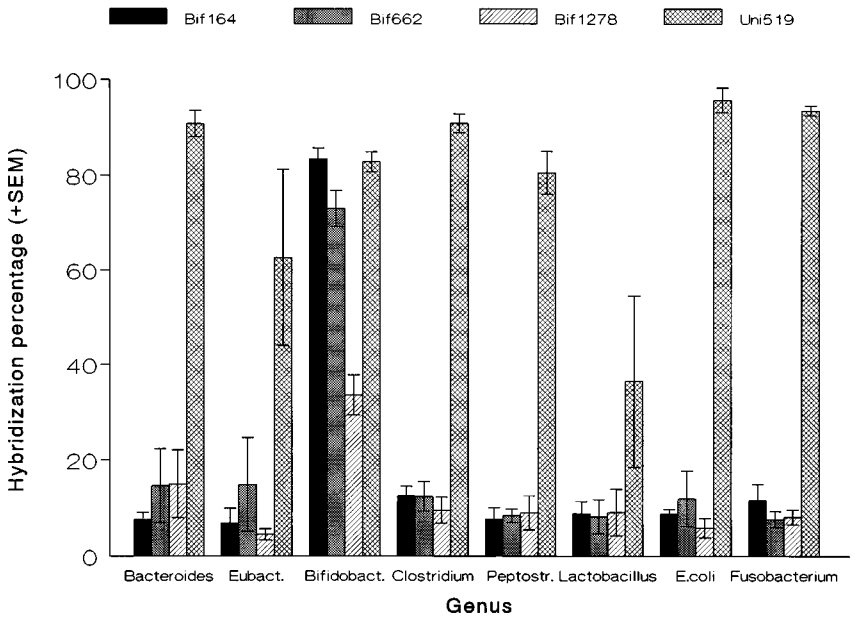

FIG. 4. Percentage of positively hybridized cells in pure cultures of intestinal bacteria after in situ hybridization with the three *Bifidobacterium*-specific probes and the universal probe. The percentages for the various genera are mean values (± SEM) for the species tested.

ples was 3.87 (± 1.73) × 10^{10} per g of wet feces. On average, bifidobacteria accounted for 6.9% ± 3.3% of the total culturable population. For all 10 individuals investigated, the total counts of fecal bacteria obtained on BBA plates were significantly lower ($P < 0.005$) than those obtained by both microscopic methods for total cell enumeration. DMCC counts, in turn, were significantly lower ($P < 0.001$) than DAPI total counts. By contrast, the number of bifidobacteria enumerated on BIF agar did not differ significantly from the microscopic count with the *Bifidobacterium*-specific probe Bif164 ($P = 0.651$). On the basis of DAPI total counts, *Bifidobacterium* spp. on average accounted for 0.8% ± 0.4% of the total population. Comparing the variances by means of the *F* test revealed that errors in the *Bifidobacterium* cultural counts and the microscopic *Bifidobacterium* counts with probe Bif164 did not differ significantly ($P < 0.05$). When comparing the methods for enumeration of total bacterial populations, the coefficient of variance for the DAPI method was lower than that for the DMCC method, which in turn was lower than that for the cultural method (Table 4).

DISCUSSION

To evaluate the performance of the probes, two parameters were determined in pure cultures of bacteria: the level of fluorescence per cell and the hybridization percentage. The first parameter reflects the amount of probe per cell. The second parameter reflects the degree of separation or overlap between the fluorescence distribution of hybridized cells and nonhybridized cells. The fact that probe Bif1278 has a minimum of three mismatches for nonbifidobacteria indicates that this probe is of potential use as a *Bifidobacterium* probe, with a high specificity for this genus. However, the performance of probe Bif1278 was inferior to those of Bif164 and Bif662 with

TABLE 4. Counts of *Bifidobacterium* spp., total bacteria, and total culturable anaerobes in fecal samples from 10 healthy human volunteers as assessed by 16S rRNA probe hybridization, direct microscopy, and cultivation on agar medium[a]

Volunteer	Bifidobacteria (10^9/g)		Total anaerobes (10^{10}/g), BBA	Total bacteria (10^{11}/g)	
	Bif164	BIF agar		DAPI	DMCC
1	2.27	1.77	7.13	2.57	1.01
2	1.58	1.22	6.15	2.50	0.88
3	0.55	ND[b]	ND	2.26	1.20
4	2.73	1.86	2.67	2.67	1.31
5	3.10	3.93	3.73	2.75	0.92
6	0.41	0.56	1.30	2.02	0.79
7	3.61	2.70	3.17	2.37	1.04
8	3.22	1.61	2.99	4.17	2.23
9	4.63	5.34	4.76	3.64	0.88
10	1.65	3.04	2.91	2.10	0.29
Mean (coefficient of variation)	2.38 (0.57)	2.45 (1.57)	3.87 (0.47)	2.71 (0.26)	1.06 (0.46)

[a] Explanation of columns: Bif164, enumeration by whole-cell hybridization with probe Bif164; BIF agar, total number of culturable bifidobacteria; BBA, total number of culturable anaerobes; DAPI, total microscopic counts with DAPI as the DNA stain; DMCC, direct microscopic clump count. All values are per gram of wet feces.
[b] ND, not determined.

respect to both the maximum attainable fluorescence level and the hybridization percentage. The mean hybridization percentage of the 16 *Bifidobacterium* spp. tested with probe Bif1278 was only 34% (Fig. 4). The poor performance of the probe may result from the presence of an intramolecular binding site in variable region V8 of the 16S rRNA, as described for *Escherichia coli* (6). Such sites may interfere with probe binding. Therefore, a theoretically suitable probe, such as Bif1278, may not be appropriate for whole-cell in situ hybridization purposes. This should be tested experimentally.

Hybridization with Uni519 revealed heterogeneity of the fluorescence level among the various species and genera. The fluorescence level for certain *Clostridium* spp. and *Fusobacterium* spp. was relatively high, while that of *Eubacterium* spp. and lactobacilli was relatively low (Fig. 3). Such differences may reflect differences in target region availability, cell permeability, or ribosome content of the cells. Low fluorescence levels in positively hybridized cells can significantly overlap high signals of the negative control (autofluorescence). By setting a threshold at the 95th percentile of the fluorescence distribution of the negative control, a discriminator for positively hybridized bacteria was obtained that ensures objective evaluation of hybridization data. However, the use of such a threshold is by no means optimal, since an overlap of the two fluorescence distributions in combination with a high fraction of potentially positive cells results in an underestimate of the hybridization percentage (23). In the case of *Eubacterium* and *Lactobacillus* spp., for example, the low levels of fluorescence after hybridization with Uni519 resulted in significant overlap with autofluorescence. This probably explains the low hybridization percentages found (cf. Fig. 3 and 4). Conversely, the hybridization percentage will be overestimated when the fraction of positive cells is low, which may explain the small but significant hybridization percentages found in nonbifidobacteria after hybridization with *Bifidobacterium*-specific probes. We are currently working on more advanced statistical analysis of these data and on specific elevation of the probe signal.

In this study, quantitative determination of hybridized bacteria in feces could not be performed on glass slides because of different adherence characteristics of the various bacterial species present in fecal samples (results not shown). Therefore, membrane filters were used for microscopic observation of cells. Since our image analysis system requires phase-contrast illumination for segmentation of bacterial objects, evaluation of hybridization with fecal samples was performed visually. According to some investigators, the majority of the fecal flora is culturable on prereduced anaerobically sterilized agar medium (7, 15, 16). In our hands, however, the culturable fraction was approximately 15% of the total DAPI counts and 37% of the DMCC counts (Table 3). This was the result not so much of low culturable counts but of higher total cell counts obtained with the DAPI method. To determine the total number of bacteria in fecal samples, DMCC (10) is routinely used in intestinal microbiology. This technique involves drying and heat fixation of cell suspensions on glass slides, followed by Gram staining, during which detachment of cells may be expected. By using epifluorescence microscopy of DAPI-stained cells filtered onto polycarbonate membranes (0.2-μm pore size), this detachment problem is prevented. Such techniques are routinely used in aquatic microbiology, for which cell populations are a millionfold below normal colon densities. We therefore believe that DAPI counts are closer to real values, which indicates that the culturability of anaerobes with the cultivation method that we have employed is low. The anaerobic cultural counts in our study do not differ significantly from values obtained by other investigators using the same techniques and media (15), but it cannot be excluded that significant oxygen damage to anaerobes can occur between defecation and anaerobic incubation of the samples. As a result, cultural techniques are prone to significant error.

The membrane filter technique was also used to enumerate the number of bacteria that could hybridize with probe Bif164. In theory, all bifidobacteria present in feces, including those that are nonculturable on agar media, should be detected by this probe hybridization technique. In analogy to the discrepancy between cultural counts and DAPI counts, it can be expected that the cultural counts of bifidobacteria are considerably lower than the probe hybridization counts. Interestingly, there was no significant difference between the number of bifidobacteria determined for any of the samples by either method. This implies not only that nearly all of the hybridized bifidobacteria were culturable, possibly as a result of their oxygen tolerance, but also that the contribution of bifidobacteria to the total intestinal microflora is largely overestimated when cultural methods are used as the sole method of enumeration. This study shows that such an overestimation can approach 10-fold. Currently we are testing genus-specific probes for detection of other major subpopulations of bacteria in human feces with the intent to perform monitoring studies on the composition and dynamics of the gut flora.

ACKNOWLEDGMENTS

We thank A. H. Weerkamp of the Netherlands Institute for Dairy Research (NIZO) and M. Dammers of the Regional Public Health Laboratory, Leeuwarden, The Netherlands, as well as G. J. Meijer-Severs and A. C. M. Wildeboer-Veloo of our department for the supply of anaerobic strains. P. Terpstra and E. Roelofsen are acknowledged for technical support.

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